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Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry

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Abstract: Anthocyanin and flavonol glycosides of various blackberry, blueberry and red wine grape genotypes were identified and measured by a high-performance liquid chromatographic (HPLC) separation method with photodiode array (PDA) and mass spectrometric (MS) detection. With this method, two distinct elution regions of anthocyanins and flavonols were obtained with near baseline separation of most compounds. Blackberry, blueberry and red wine grape genotypes varied markedly in total anthocyanins and total flavonols as well as oxygen radical absorbance capacity (ORAC). The respective ranges of total anthocyanin (TA) and total flavonol (TF) contents of tested samples were: blackberries, 1143.9–2415.4 and 102.0–160.2 mg kg^{-1}; blueberries, 1435.2–8227.3 and 172.5–327.5 mg kg^{-1}; and red wine grapes, 380.9–7904.7 and 21.0–322.2 mg kg^{-1}. Antioxidant activities and contents of total anthocyanins and total flavonols in blackberries, blueberries and red wine grapes were highly correlated, with linear relationships between ORAC and TA ($r_{xy} = 0.94$) and TF ($r_{xy} = 0.90$) for grapes, TA ($r_{xy} = 0.95$) for blueberries and TA ($r_{xy} = 0.74$) for blackberries.

Keywords: flavonols; anthocyanins; antioxidant capacity (ORAC); blackberry; blueberry; red grape; HPLC/MS

INTRODUCTION

Flavonoids have a wide range of biological effects *in vitro*, including antioxidant, anti-inflammatory, antiallergic, antiulcer, antibiotic and anticarcinogenic properties.[1] Epidemiological evidence suggests that high consumption of plant-derived flavonoids may provide protection against coronary heart disease,[2–5] stroke[6] and lung cancer.[7] A major role of flavonoids in both plants and humans is protection against oxidative stress promoted by free radical species. Since oxidative stress plays a major role in many chronic diseases,[8] it is thought that increased consumption of flavonoid-rich foods may reduce the incidence and mortality rates of chronic diseases.

Flavonoids are polyphenolic compounds that constitute a large group of secondary plant metabolites. The predominant flavonoids found in berries and red grapes are the anthocyanins and flavonols, which are almost exclusively present in glycosylated forms. The anthocyanins can also exist as diglycosides or as acylated forms of glycosides. Owing to the large number of flavonoid glycosides present in fruits and the lack of analytical standards available for many compounds, many investigators have used acid hydrolysis techniques to cleave glycosidic bonds, followed by HPLC analysis to quantify the aglycones. Although this method simplifies the identification of flavonoids and provides important information concerning total amounts of individual flavonoid aglycones in foods, it does not reflect the authentic structure of flavonoid glycosides consumed in the diet. Antioxidant capacity of flavonoids is influenced by type of sugar moiety,[9] degree of glycosylation[9] as well as acylation of anthocyanin glucosides.[10,11] The sugar moiety of quercetin glycosides is an important factor affecting their absorption and bioavailability *in vivo*.[12] A further limitation associated with acid hydrolysis of flavonoid glycosides is that acid concentrations and incubation times and temperatures need to be optimised for different classes of flavonoids.[13] Ideally, flavonoids should be analysed in their native form as glycosides in order to identify structure–activity

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relationships related to antioxidant capacity as well as other biological functions.

The objective of this work was to develop a reverse phase HPLC method coupled with photodiode array and mass spectrometric detection to separate, identify and quantify flavonoid glycosides in different genotypes of blackberries, blueberries and red wine grapes within a single run, and observe the relationship between antioxidant activity and levels of total anthocyanins and total flavonols.

EXPERIMENTAL

Chemicals

A standard mixture containing 3-*o*-monoglucosides of cyanidin (Cyd), delphinidin (Dpd), malvidin (Mvd), peonidin (Pnd) and petunidin (Ptd) was obtained from Polyphenols Laboratories AS (Sandnes, Norway). Quercetin 3-glucoside and quercetin 3-galactoside were obtained from Indofine Chemical Company (Somerville, NJ, USA). Chlorogenic acid, fluorescein and rutin were obtained from Sigma Chemical Company (St Louis, MO, USA). 2,2′-Azobis(2-amidino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals, Inc (Richmond, VA, USA) and Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) was obtained from Aldrich (Milwaukee, WI, USA). HPLC-grade methanol was obtained from JT Baker Inc (Phillipsburg, NJ, USA) and formic acid was obtained from Burdick & Jackson (Muskegon, MI, USA).

Samples

Fully mature fruits from five genotypes of blueberries and red wine grapes and six genotypes of blackberries (floricane fruits only were utilised, including that for APF-12) were harvested and stored at −20 °C until analysis. Blueberry genotypes analysed included two commercial cultivars, Bluecrop (*Vaccinium corymbosum* L with common name northern highbush type) and Ozarkblue (a hybrid of majority *V corymbosum* germplasm with some contribution from *V darrowi* L and *V ashei* Reade and in the group of blueberries knows as southern highbush), and three advanced breeding selections, A-98, US-497 and US-720, that were small-fruited genotypes with exceptionally dark skin colour. Selection A-98 is a 3/4 *V corymbosum* 1/4 *V darrowi* hybrid, while US-497 and US-720 have as common parents in their backgrounds the species *V corymbosum, V ashei, V darrowi* and *V atrococcum* Heller and are rather unique hybrids in blueberry breeding. The blackberry genotypes analysed included five commercial cultivars, Apache, Arapaho, Chickasaw, Kiowa and Navaho, and one advanced breeding selection, APF-12. The blackberries are considered as *Rubus* subgenus *Rubus* Watson, as a species classification is not possible owing to their broad genetic background of several native North American species (the major one *R allegheniensis* Porter) along with some other species which contribute the gene for thornlessness (Apache, Arapaho and Navaho). They are derived largely from the same original parent material and as a group do not vary greatly in overall genetic background. Red wine grape genotypes analysed included two commercial cultivars, Cynthiana (*Vitis aestivalis* Michx) and Cabernet-Sauvignon (*V vinifera* L), and three advanced wine grape breeding selections, A-1575, A-2467 and A-2633, that had exceptionally dark skin colour as well as pigmentation throughout the flesh. Except for A-2467, which is completely *V vinifera* derived from several cultivars, the breeding selections have a mixed species background and differ so to some extent from the named cultivars. Selection A-1575 is a hybrid of the *V vinifera* cultivar Royalty × A-1173 (a hybrid of Lomanto, which is a cultivar released by TV Munson in Texas and may have several native American species in its background, × V 49432, which is a French–American hybrid); A-2633 is a very unique combination of *V vinifera* plus several other germplasm sources, including American species hybrid selections from New York, Mississippi and Florida.

Analyses

Extraction

Seeds were removed from red wine grapes prior to extraction. Frozen fruits were blended to a puree using a Black & Decker Handy Chopper Plus commercial blender. Subsamples (5 g) of puree were then homogenised for 1 min in 20 ml of extraction solution containing methanol/water/formic acid (60:37:3 v/v/v) to the smallest particle size using a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corp, Mason, OH, USA). Homogenates were filtered through Miracloth (CalBiochem, LaJolla, CA, USA) and the filtrates were centrifuged for 5 min at 5000 rpm. Aliquots (4 ml) of supernatant were evaporated to dryness using a SpeedVac® concentrator (ThermoSavant, Holbrook, NY, USA) with no radiant heat and resuspended in 1 ml of aqueous 3% formic acid solution. In preliminary studies we had found that application of radiant heat during the concentration step resulted in significant degradation of flavonoid glycosides. All samples were passed through 0.45 μm filters (Whatman) prior to HPLC analysis. Triplicate extractions were prepared from each fruit genotype.

HPLC analysis of flavonoids

Samples (50 μL) were analysed using a Waters HPLC system equipped with a model 600 pump, a model 717 Plus autosampler and a model 996 photodiode array detector. Separation was carried out using a 4.6 mm × 250 mm Symmetry® C18 column (Waters Corp, Milford, MA, USA) with a 3.9 mm × 20 mm Symmetry® C18 guard column. The mobile phase was a linear gradient of 5% formic acid (A) and methanol (B) from 2% B to 60% B for 60 min at 1 ml min^{-1}. The system was equilibrated for 20 min at the initial gradient prior to each injection. Detection wavelengths

used were 360 nm for flavonols and 510 nm for anthocyanins. Individual anthocyanin monoglucosides and acylated anthocyanin derivatives were quantified as Dpd, Cyd, Ptd, Pnd and Mvd glucoside equivalents. Total anthocyanins were calculated as the sum of individual anthocyanin monoglucosides and acylated anthocyanin derivatives. Flavonols were quantified as rutin equivalents, and chlorogenic acid in blueberries was quantified using an authentic standard.

HPLC/ESI-MS analysis of flavonoids

An analytical Hewlett Packard 1100 series HPLC instrument equipped with an autosampler, a binary HPLC pump and a UV/VIS detector was used. Reverse phase separations of flavonoids were performed using the same HPLC conditions as described above, with absorption at 360 nm recorded for flavonols and 510 nm for anthocyanins. For HPLC/MS analysis the HPLC apparatus was interfaced to a Bruker Esquire LC/MS ion trap mass spectrometer. Mass spectral data were collected with the Bruker software, which also controlled the instrument and collected the signal at 360 or 510 nm. Typical conditions for mass spectral analysis in positive ion electrospray mode for anthocyanins and negative ion electrospray mode for flavonols included a capillary voltage of 4000 V, a nebulising pressure of 30.0 psi, a drying gas flow of 9.0 ml min^{-1} and a temperature of 300 °C. Data were collected in full scan mode over a mass range of m/z 50–1000 at 1.0 s per cycle. Characteristic ions were used for peak assignment (Table 1). For compounds where chemical standards were commercially available, retention times were also used to confirm the identification of components.

Table 1. Peak assignments, retention times (RT) and mass spectral data of anthocyanins and flavonols detected in red grape, blackberry and blueberry genotypes

Peak	HPLC RT (min)	Identification		(m/z) values
		Chlorogenic acid	M^-	*Fragments*
1	24.6	Chlorogenic acid	353	191
		Anthocyanins	M^+	*Fragments*
2	28.2	Delphinidin 3-galactoside	465	303
3	29.7	Delphinidin 3-glucoside	465	303
4	30.5	Cyanidin 3-galactoside	449	287
5	31.4	Delphinidin 3-arabinoside	435	303
6	32.3	Cyanidin 3-glucoside	449	287
7	33.0	Petunidin 3-galactoside	479	317
8	33.7	Cyanidin 3-arabinoside	419	287
9	34.3	Petunidin 3-glucoside	479	317
10	34.8	Peonidin 3-galactoside	463	301
11	35.9	Petunidin 3-arabinoside	449	317
12	36.4	Malvidin 3-galactoside	493	331
13	36.7	Peonidin 3-glucoside	463	301
14	37.8	Malvidin 3-glucoside	493	331
15	38.5	Peonidin 3-arabinoside	433	301
16	39.5	Malvidin 3-arabinoside	463	331
17	43.2	Delphinidin 3-acetylglucoside	507	303
18	45.6	Cyanidin 3-acetylglucoside	491	287
19	46.8	Petunidin 3-acetylglucoside	521	317
20	49.4	Malvidin 3-acetylglucoside	535	331
21	51.4	Delphinidin 3-(*p*-coumaroyl)glucoside	611	303
22	53.4	Cyanidin 3-(*p*-coumaroyl)glucoside	595	287
23	54.2	Petunidin 3-(*p*-coumaroyl)glucoside	625	317
24	56.1	Malvidin 3-(*p*-coumaroyl)glucoside	639	331
25	33.9	Cyanidin 3-rutinoside	595	449, 287
26	40.2	Cyanidin 3-xyloside	419	287
27	41.5	Cyanidin 3-malonylglucoside	535	287
28	43.0	Cyanidin 3-dioxalylglucoside	593	477, 361, 287, 133
		Flavonols	M^-	*Fragments*
29	40.2	Myricetin 3-galactoside/glucoside	479	317
30	42.8	Myricetin 3-rhamnoside	463	317
31	44.5	Quercetin 3-galactoside	463	301
32	45.2	Quercetin 3-glucoside	463	301
33	45.4	Quercetin 3-rutinoside	609	463, 301
34	47.0	Quercetin 3-xyloside	433	301
35	47.3	Quercetin 3-xylosylglucuronide (tentative)	607	301
36	48.8	Quercetin 3-glucosylxyloside	595	433, 301
37	49.3	Quercetin 3-acetylrhamnoside	489	447, 301

Determination of antioxidant capacity

The oxygen radical absorbance capacity (ORAC) of extracts was measured using the method of Prior *et al*[14] modified for use with a FLUOstar Optima microplate reader (BMG Labtechnologies, Durham, NC, USA) using fluorescein as fluorescent probe. Fruit extracts were diluted 1600-fold or more with phosphate buffer (75 mM, pH 7) prior to ORAC analysis. The assay was carried out in clear 48-well Falcon plates (VWR, St Louis, MO, USA). Each well had a final volume of 590 μl. Initially, 40 μl of diluted sample, Trolox (TE) standards (6.25, 12.5, 25, 50 μM) and blank solution (75 mM, pH 7 phosphate buffer) were added to each well using an automatic pipette. The FLUOstar Optima instrument equipped with two automated injectors was then programmed to add 400 μl of fluorescein (0.108 μM) followed by 150 μl of AAPH (31.6 mM) to each well. Fluorescence readings (excitation 485 nm, emission 520 nm) were recorded after the addition of fluorescein, after the addition of AAPH and every 192 s thereafter for 112 min to reach 95% loss of fluorescence. Final fluorescence measurements were expressed relative to the initial reading. Results were calculated based upon differences in areas under the fluorescein decay curve between the blank, samples and standards. The standard curve was obtained by plotting the four concentrations of TE against the net area under the curve (AUC) of each standard. Final ORAC values were calculated using the regression equation between TE concentration and AUC and are expressed as mmol TE equivalents kg^{-1} fresh weight.

Statistical analysis

Analysis of variance[15] was used to determine significant differences ($P < 0.05$) in total flavonoid and total anthocyanin contents and antioxidant capacity among genotypes of each fruit analysed. The relationships between ORAC values and contents of flavonols and anthocyanins in the fruits were determined using the Pearson correlation test.

RESULTS AND DISCUSSION

HPLC method

The gradient method developed in combination with the Symmetry® C_{18} column provided excellent separation of anthocyanins in red wine grapes, blueberries and blackberries (Fig 1). The HPLC profile of anthocyanins in the red wine grape genotype A-1575 is shown in Fig 1(A). Similar separation of anthocyanins was obtained for the other five red wine grape genotypes. Peaks 3, 6, 9, 13 and 14 represent the 3-monoglucosides of Dpd, Cyd, Ptd, Pnd and Mvd respectively. Peaks 17–20 were identified as acetylated derivatives of the 3-monoglucosides of Dpd, Cyd, Ptd and Mvd respectively. Peaks 21–24 were identified as *p*-coumaroyl derivatives of the 3-monoglucosides of Dpd, Cyd, Ptd and Mvd respectively. All the compounds detected in the red wine grapes have previously been identified by LC/MS.[16,17] The HPLC profiles can clearly be used as a tool to differentiate variation in anthocyanin components among genotypes. For example, A-2633 and Cynthiana contained a large Cyd 3-glucoside peak that clearly differed from the other genotypes in which Mvd 3-glucoside was the largest peak (data not shown).

The HPLC profile of anthocyanins in Bluecrop blueberry is shown in Fig 1(B). Achieving baseline separation of all the anthocyanins present in blueberries is challenging, since the fruit contains galactosides, glucosides and arabinosides of Dpd, Cyd, Ptd, Pnd and Mvd. The elution orders and mass spectral data of the 14 monomeric anthocyanin glycosides detected were in good agreement with previous studies.[18,19] Although anthocyanin glucosides and galactosides have identical masses, the galactosides elute before the glucosides on a C_{18} column.[18] Peaks 17, 19 and 20 were identified as acetylated derivatives of Dpd 3-glucoside, Ptd 3-glucoside and Mvd 3-glucoside respectively.

The HPLC profile of anthocyanins in Kiowa blackberry is shown in Fig 1(C). Relative to red wine grapes and blueberries, the anthocyanin profile of blackberries was simple, with only five anthocyanins detected. Cyd 3-glucoside (peak 6) was the predominant anthocyanin in blackberries. The other minor anthocyanins identified included Cyd 3-rutinoside (peak 25), Cyd 3-xyloside (peak 26), Cyd 3-malonylglucoside (peak 27) and Cyd 3-dioxalylglucoside (peak 28). The identification of anthocyanins in blackberries was consistent with previous reports.[20–23]

The HPLC profile of flavonols detected at 360 nm in the red wine grape genotype A-1575 is shown in Fig 2(A). Although six flavonols were detected in A-1575, only myricetin 3-galactoside/glucoside (peak 29), quercetin 3-galactoside (peak 31) and quercetin 3-glucoside (peak 32) were positively identified. Although quercetin 3-galactoside and quercetin 3-glucoside have identical *m/z* values, we confirmed that the galactoside eluted before the glucoside using authentic standards of the two compounds.

The flavonols in Bluecrop blueberry were identified as myricetin 3-galactoside/glucoside (peak 29), myricetin 3-rhamnoside (peak 30), quercetin 3-galactoside (peak 31), quercetin 3-glucoside (peak 32), quercetin 3-rutinoside (peak 33) and quercetin 3-acetylrhamnoside (peak 37) (Fig 2(B)). Kader *et al*[24] previously identified the main flavonol glycosides in highbush blueberries as quercetin 3-glucoside, quercetin 3-galactoside and quercetin 3-rhamnoside, which concurs with our findings, except that quercetin 3-rhamnoside appeared to be acetylated in the blueberries we studied. Seven additional flavonols were present that we were unable to identify. The blueberry extract also contained a large early eluting peak that was identified as chlorogenic acid (peak 1).

The flavonols in Kiowa blackberry were identified as quercetin 3-galactoside (peak 31), quercetin

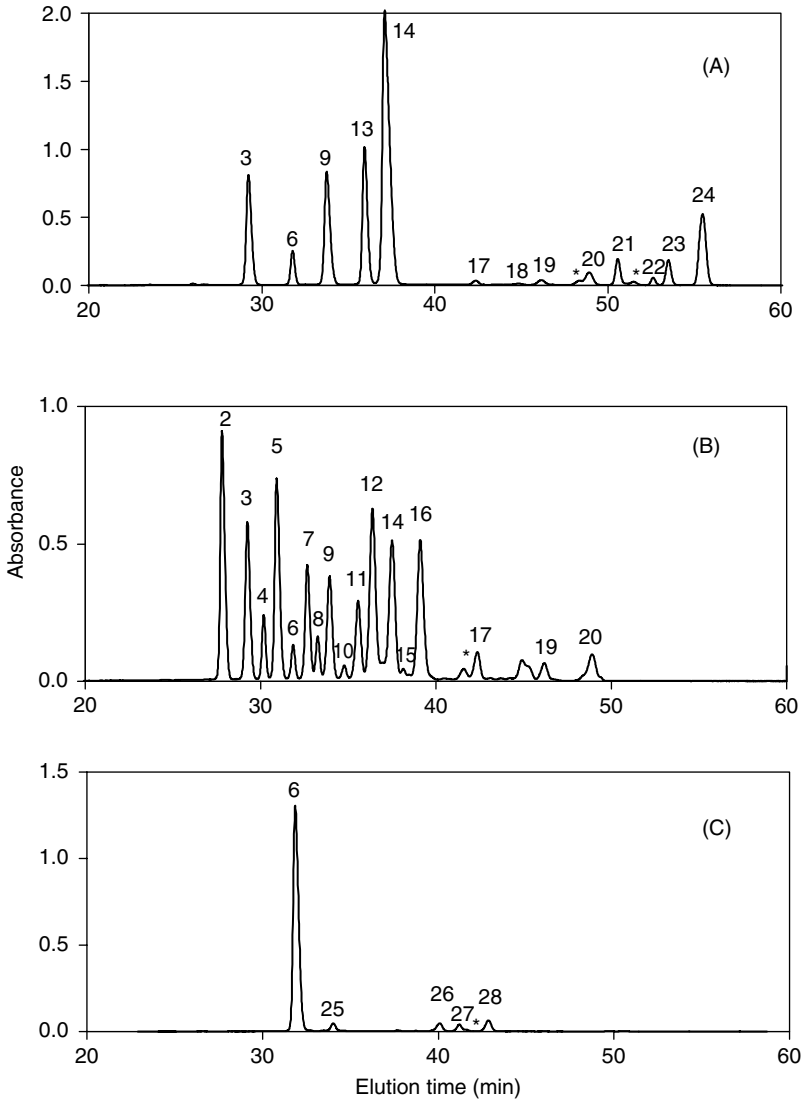

Figure 1. Detection of anthocyanin glycosides (510 nm) in (A) A-1575 red wine grape, (B) Bluecrop blueberry and (C) Kiowa blackberry. See Table 1 for peak identification. *Unidentified anthocyanins.

3-glucoside (peak 32), quercetin 3-rutinoside (peak 33), quercetin 3-xyloside (peak 34), quercetin 3-xylosylglucuronide (peak 35, tentative) and quercetin 3-glucosylxyloside (peak 36) (Fig 2(C)). The identification of flavonols in blackberries was consistent with previous reports,[22,25] except for quercetin 3-glucosylxyloside which has not previously been identified. Henning[25] previously identified kaempferol 3-glucuronide, kaempferol 3-glucoside, kaempferol 3-galactoside and kaempferol 3-xylosylglucuronide in blackberries, but no kaempferol glycosides were detected in the genotypes we analysed. This discrepancy may be attributed to differences in genetics or sample concentration prior to analysis.

Flavonoid components and antioxidant activities in grapes

The total anthocyanin content of the five red wine grape genotypes ranged from a low of 380.9 mg kg^{-1} for Cabernet-Sauvignon to a high of 7904.7 mg kg^{-1} for A-2467, reflecting a 21-fold difference in total anthocyanin content among genotypes (Table 2). A-1575 (7331.3 mg kg^{-1}) and A-2633 (3460.5 mg kg^{-1}) also contained high levels of total anthocyanins compared with Cynthiana and Cabernet-Sauvignon. Mvd 3-glucoside was the predominant anthocyanin in A-1575, A-2467 and Cabernet-Sauvignon, while Cyd 3-glucoside was the predominant anthocyanin in A-2633 and Cynthiana. In addition to Mvd 3-glucoside, the genotypes contained appreciable levels of Ptd, Pnd and Dpd 3-glucosides. Typically, the anthocyanins that were present in large amounts as monomeric glucosides were also present as acylated forms in lesser amounts. A-2467 contained the highest amounts of Dpd 3-acetylglucoside, Ptd 3-acetylglucoside, Mvd 3-acetylglucoside and Mvd 3-(*p*-coumaroyl) glucoside. The composition and predominance of anthocyanins in Cabernet-Sauvignon were similar to previous reports.[26,27]

The percentage distribution of anthocyanins in the five genotypes of red wine grapes is presented in Table 3. All the red wine grape genotypes contained monoglucosides of Dpd, Cyd, Ptd, Pnd

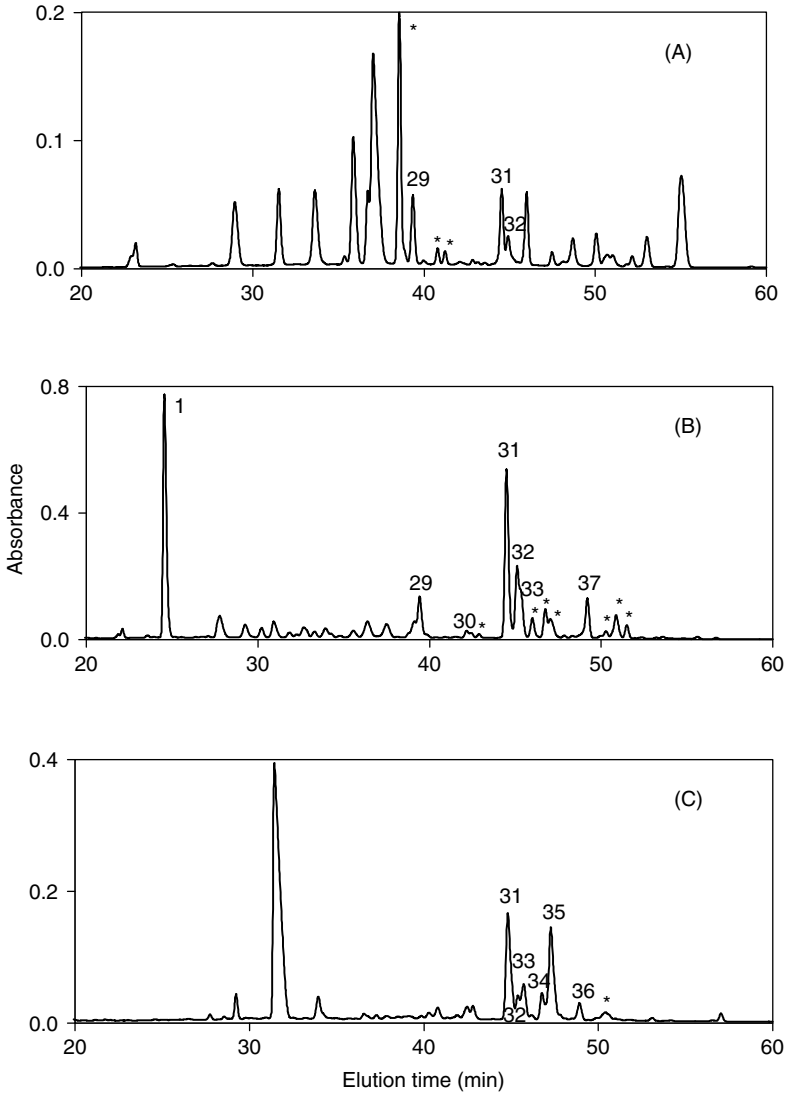

Figure 2. Detection of flavonol glycosides (360 nm) in (A) A-1575 red wine grape, (B) Bluecrop blueberry and (C) Kiowa blackberry. See Table 1 for peak identification. *Unidentified flavonols.

Table 2. Anthocyanin content (mg kg^{-1} fresh weight) of red wine grape genotypes

	Genotype				
Compound	A-1575[a]	A-2467[a]	A-2633[a]	Cynthiana	Cabernet-Sauvignon
Delphinidin 3-glucoside	870.7 ± 48.6[d]	770.7 ± 24.2	154.5 ± 34.4	166.1 ± 29.0	21.0 ± 1.4
Cyanidin 3-glucoside	176.7 ± 6.8	220.9 ± 8.4	902.9 ± 44.8	187.2 ± 10.4	7.3 ± 0.9
Petunidin 3-glucoside	1106.5 ± 48.1	966.7 ± 22.9	175.1 ± 33.6	62.0 ± 8.6	12.1 ± 1.3
Peonidin 3-glucoside	700.7 ± 7.1	866.7 ± 18.2	46.5 ± 9.3	13.4 ± 2.3	33.1 ± 2.4
Malvidin 3-glucoside	2767.3 ± 41.3	2284.0 ± 89.1	251.3 ± 34.7	69.5 ± 4.8	164.7 ± 9.0
Delphinidin 3-acetylglucoside	43.7 ± 2.1	241.7 ± 8.3	ND	20.6 ± 4.4	5.4 ± 0.2
Cyanidin 3-acetylglucoside	15.7 ± 1.5	88.1 ± 13.8	179.2 ± 26.8	39.6 ± 8.9	ND
Petunidin 3-acetylglucoside	34.0 ± 3.7	330.8 ± 6.1	255.1 ± 40.4	24.3 ± 5.8	ND
Malvidin 3-acetylglucoside	151.4 ± 3.7	650.7 ± 34.5	599.0 ± 87.6	64.4 ± 6.5	96.6 ± 1.8
Delphinidin 3-(*p*-coumaroyl)glucoside	258.5 ± 16.0	250.7 ± 27.7	178.3 ± 33.3	61.9 ± 17.2	ND
Cyanidin 3-(*p*-coumaroyl)glucoside	60.1 ± 2.8	64.9 ± 2.4	12.2 ± 1.1	9.4 ± 2.4	2.6 ± 0.2
Petunidin 3-(*p*-coumaroyl)glucoside	266.0 ± 15.1	210.4 ± 15.8	133.4 ± 23.3	15.2 ± 5.1	0.5 ± 0.1
Malvidin 3-(*p*-coumaroyl)glucoside	880.0 ± 32.4	958.4 ± 15.5	204.0 ± 33.7	24.4 ± 3.6	37.6 ± 4.6
Unknown anthocyanins[b]	ND	ND	369.0 ± 47.7	133.7 ± 22.0	ND
Total anthocyanins[c]	7331.3b	7904.7a	3460.5c	891.7d	380.9e

[a] Breeding selection not available for sale or present in commerce at the time of writing.
[b] Data expressed as cyanidin 3-monoglucoside equivalents.
[c] Values with similar letters are not significantly different (LSD, $P > 0.05$).
[d] Standard deviation ($n = 3$). ND, not detected.

Table 3. Percentage distribution of monomeric and acylated anthocyanins[a] in red wine grape genotypes

	Monomeric					Acylated				
Genotype	Dpd	Cyd	Ptd	Pnd	Mvd	Dpd	Cyd	Ptd	Mvd	Unk[b]
A-1575[c]	11.9	2.4	15.1	9.6	37.7	4.1	1.0	3.6	12.0	0.0
A-2467[c]	9.7	2.8	12.2	11.0	28.9	6.3	1.9	6.9	20.3	0.0
A-2633[c]	4.5	26.1	5.1	1.3	7.3	5.2	5.6	11.3	23.2	10.7
Cynthiana	18.6	21.0	7.0	1.5	7.8	9.2	5.5	4.4	9.9	15.0
Cabernet-Sauvignon	5.5	1.9	3.2	8.7	43.2	1.4	0.7	0.1	35.3	0.0

[a] Dpd, delphinidin; Cyd, cyanidin; Ptd, petunidin; Pnd, peonidin; Mvd, malvidin.
[b] Unknown peak expressed as cyanidin 3-monoglucoside equivalents.
[c] Breeding selection not available for sale or present in commerce at the time of writing.

Table 4. Flavonol content (mg kg^{-1} fresh weight[a]) and oxygen radical absorbance capacity ($ORAC_{FL}$) (mmol TE kg^{-1} fresh weight) of red wine grape genotypes

	Genotype				
Compound/ORAC	A-1575[b]	A-2467[b]	A-2633[b]	Cynthiana	Cabernet-Sauvignon
Myricetin 3-galactoside/glucoside	35.9 ± 0.2[d]	101.9 ± 6.8	43.0 ± 5.4	10.1 ± 1.2	6.5 ± 1.0
Quercetin 3-galactoside	35.7 ± 4.7	ND	56.5 ± 4.2	8.5 ± 1.0	9.4 ± 0.5
Quercetin 3-glucoside	19.0 ± 1.1	ND	ND	ND	ND
Unknown flavonols	102.1 ± 29.3	220.3 ± 24.9	19.1 ± 2.5	2.4 ± 0.3	15.3 ± 3.5
Total flavonols[c]	192.7b	322.2a	118.6c	21.0d	31.2d
ORAC[c]	116.6b	135.8a	97.1c	42.8d	37.2d

[a] Data expressed as rutin equivalents.
[b] Breeding selection not available for sale or present in commerce at the time of writing.
[c] Values within a row with similar letters are not significantly different (LSD, $P > 0.05$).
[d] Standard deviation ($n = 3$). ND, not detected.

and Mvd and acylated derivatives of Dpd, Cyd, Ptd and Mvd. Malvidin was the predominant monoglucoside in A-1575, A-2467 and Cabernet-Sauvignon, accounting for 37.7, 28.9 and 43.2% of total anthocyanins respectively. In contrast, cyanidin was the predominant monoglucoside in A-2633 and Cynthiana, accounting for 26.1 and 21.0% of total anthocyanins respectively (Table 3). The higher percentage of cyanidin monoglucoside in these two genotypes is likely due to the substantial species background differences between these two and the other genotypes measured. A-2467, A-2633 and Cabernet-Sauvignon contained high levels of acylated derivatives of Mvd, accounting for 20.3, 23.2 and 35.3% of total anthocyanins respectively. The higher percentage of acylated anthocyanins in these genotypes indicates that they may confer greater colour stability in wines. Cabernet-Sauvignon was unique in that the sum of monoglucosides and acylated derivatives of Mvd accounted for 78.5% of total anthocyanins.

The five red wine grape genotypes contained much lower levels of total flavonols compared with anthocyanins, ranging from a low of 21.0 mg kg^{-1} for Cynthiana to a high of 322.2 mg kg^{-1} for A-2467 (Table 4). A-2467 contained higher levels of myricetin 3-galactoside/glucoside and unknown flavonols than the other genotypes, while A-2633 contained the highest level of quercetin 3-galactoside.

ORAC values of red wine grapes ranged from a low of 37.2 mmol TE kg^{-1} for Cabernet-Sauvignon to a high of 135.8 mmol TE kg^{-1} for A-2467, reflecting a 3.6-fold difference in antioxidant capacity among genotypes (Table 4). A-1575 (116.6 mmol TE kg^{-1}) and A-2633 (97.1 mmol TE kg^{-1}) also had exceptionally high ORAC values. Linear relationships were observed between ORAC values and total anthocyanin monoglucosides ($r_{xy} = 0.78$), total acylated anthocyanins ($r_{xy} = 0.84$), total anthocyanins ($r_{xy} = 0.94$) and total flavonols ($r_{xy} = 0.90$). Although the positive relationship between ORAC and levels of total phenolics and anthocyanins in fruits is well established,[28,29] the higher correlation observed between ORAC and total acylated anthocyanins compared with ORAC and total monoglucosides is interesting and indirectly confirms studies reporting that acylated anthocyanins have greater antioxidant capacity than monoglucosides.[10,11]

Flavonoid components and antioxidant activities in blueberries

The contents of the 14 anthocyanin monoglycosides, three acylated anthocyanins and total anthocyanins in the five blueberry genotypes are shown in Table 5. The total anthocyanin content of blueberry genotypes ranged from a low of 1435.2 mg kg^{-1} for Bluecrop to a high of 8227.3 mg kg^{-1} for US-497, reflecting a 5.7-fold difference in total anthocyanin content among genotypes. US-720 (4319.4 mg kg^{-1}) and A-98 (3691.2 mg kg^{-1}) also contained exceptionally high

Table 5. Anthocyanin content (mg kg^{-1} fresh weight) of blueberry genotypes

	Genotype				
Compound	A-98[a]	Bluecrop	Ozarkblue	US-497	US-720
Delphinidin 3-galactoside	730.5 ± 56.1[d]	188.5 ± 22.1	389.8 ± 41.2	1519.0 ± 92.6	973.1 ± 88.2
Delphinidin 3-glucoside	14.3 ± 1.2	126.4 ± 15.2	8.1 ± 0.8	50.4 ± 4.2	28.2 ± 3.8
Cyanidin 3-galactoside	155.1 ± 8.8	35.6 ± 3.4	112.7 ± 12.3	762.5 ± 57.0	319.8 ± 33.3
Delphinidin 3-arabinoside	362.8 ± 40.8	163.1 ± 19.7	185.8 ± 17.1	659.8 ± 49.4	540.0 ± 76.6
Cyanidin 3-glucoside	7.1 ± 0.8	20.2 ± 2.0	4.0 ± 0.4	38.0 ± 1.6	13.0 ± 1.4
Petunidin 3-galactoside	544.5 ± 48.5	113.3 ± 14.5	228.6 ± 23.8	1133.2 ± 109.6	766.4 ± 85.7
Cyanidin 3-arabinoside	64.2 ± 5.0	26.1 ± 2.6	46.7 ± 4.7	349.4 ± 29.2	137.5 ± 18.8
Petunidin 3-glucoside	14.3 ± 1.0	111.4 ± 14.2	7.5 ± 0.9	57.4 ± 4.2	33.6 ± 4.0
Peonidin 3-galactoside	58.7 ± 3.6	13.8 ± 0.9	18.4 ± 2.1	335.8 ± 22.4	62.4 ± 6.8
Petunidin 3-arabinoside	243.5 ± 23.5	81.9 ± 12.1	113.7 ± 10.2	365.9 ± 52.6	330.1 ± 40.5
Malvidin 3-galactoside	715.6 ± 49.2	159.8 ± 25.0	195.7 ± 20.9	1792.6 ± 83.8	681.3 ± 80.3
Malvidin 3-glucoside	43.8 ± 2.4	153.3 ± 22.0	18.7 ± 2.3	195.9 ± 20.9	67.0 ± 6.8
Peonidin 3-arabinoside	68.2 ± 9.6	6.4 ± 0.5	2.4 ± 0.8	34.8 ± 2.5	ND
Malvidin 3-arabinoside	352.1 ± 25.6	144.0 ± 22.0	105.2 ± 9.5	718.6 ± 66.7	357.0 ± 41.8
Delphinidin 3-acetylglucoside	68.7 ± 8.6	27.9 ± 4.1	5.3 ± 1.2	44.2 ± 3.8	6.4 ± 0.3
Petunidin 3-acetylglucoside	190.9 ± 20.2	15.8 ± 3.3	ND	114.9 ± 9.9	ND
Malvidin 3-acetylglucoside	17.4 ± 1.5	36.1 ± 4.6	ND	10.0 ± 0.5	ND
Unknown acylated anthocyanins[b]	39.5 ± 4.7	11.6 ± 2.2	1.3 ± 0.1	44.9 ± 4.2	3.6 ± 0.3
Total anthocyanins[c]	3691.2b	1435.2c	1443.9c	8227.3a	4319.4b

[a] Breeding selection not available for sale or present in commerce at the time of writing.
[b] Data expressed as delphinidin 3-monoglucoside equivalents.
[c] Values with similar letters are not significantly different (LSD, $P > 0.05$).
[d] Standard deviation ($n = 3$). ND, not detected.

Table 6. Percentage distribution of monomeric and acylated anthocyanins[a] and anthocyanin glycosides in blueberry genotypes

	Monomeric						Glycoside		
Genotype	Dpd	Cyd	Ptd	Pnd	Mvd	Acylated	Galactoside	Glucoside	Arabinoside
A-98[b]	30.0	6.1	21.7	3.4	30.1	8.6	59.7	2.2	29.6
Bluecrop	33.3	5.7	21.4	1.4	31.8	6.4	35.6	28.7	29.3
Ozarkblue	40.4	11.3	24.2	1.4	22.1	0.5	65.5	2.7	31.4
US-497	27.1	14.0	18.9	4.5	32.9	2.6	67.4	4.2	25.9
US-720	35.7	10.9	26.2	1.4	25.6	0.2	64.9	3.3	31.6

[a] Dpd, delphinidin; Cyd, cyanidin; Ptd, petunidin; Pnd, peonidin; Mvd, malvidin.
[b] Breeding selection not available for sale or present in commerce at the time of writing.

levels of total anthocyanins. The total anthocyanin content of Bluecrop (1435.2 mg kg^{-1}) was higher than the two values (1090–1120 mg kg^{-1}) reported by Gao and Mazza,[30] which may be attributed to differences in environmental growing conditions.

Generally, the small-fruited genotypes A-98, US-497 and US-720 had much higher levels of anthocyanin monoglycosides than the large-fruited genotypes Bluecrop and Ozarkblue. US-497 in particular had exceptionally high levels of Mvd 3-galactoside, Dpd 3-galactoside, Cyd 3-galactoside, Ptd 3-galactoside and Mvd 3-arabinoside compared with the other genotypes. A-98 was unique in that it contained the highest amounts of Dpd 3-acetylglucoside and Ptd 3-acetylglucoside.

The percentage distribution of anthocyanins in the five genotypes of blueberries is presented in Table 6. Although the amounts of anthocyanins in the five genotypes varied markedly, the relative distribution of anthocyanins was similar. Dpd (27.1–40.4%), Mvd (22.1–32.9%) and Ptd (18.9–26.2%) were the predominant monomeric anthocyanins, followed by Cyd (5.7–14.0%) and Pnd (1.4–4.5%). Monomeric anthocyanins accounted for 91–99% of total anthocyanins in blueberries, reflecting a minor contribution by acylated anthocyanins. A-98 and Bluecrop were the only two genotypes that had appreciable levels, 8.6 and 6.4% respectively, of acylated anthocyanins. The percentage distribution of anthocyanins in Bluecrop (Dpd 33.3%, Cyd 5.7%, Ptd 21.4%, Pnd 1.4% and Mvd 31.8%) agreed well with values reported by Skrede *et al*[19] (Dpd 26%, Cyd 11%, Ptd 17%, Pnd 2% and Mvd 44%) and Kalt *et al*[31] (Dpd 41.1%, Cyd 5.8%, Ptd 19.4%, Pnd 1.3% and Mvd 32.1%) for Bluecrop.

The percentage distribution of glycosides showed that galactosides accounted for 59.7–67.4%, arabinosides 29.6–31.6% and glucosides 2.2–4.2% in

Table 7. Flavonol content (mg kg^{-1} fresh weight[a]) and oxygen radical absorbance capacity ($ORAC_{FL}$) (mmol TE kg^{-1} fresh weight) of blueberry genotypes

	Genotype				
Compound/ORAC	A-98[b]	Bluecrop	Ozarkblue	US-497	US-720
Myricetin 3-galactoside/glucoside	38.4 ± 6.0[d]	22.8 ± 1.4	25.0 ± 1.0	40.2 ± 2.2	22.5 ± 4.4
Myricetin 3-rhamnoside	7.7 ± 1.1	6.4 ± 0.7	14.3 ± 0.5	4.7 ± 0.4	3.4 ± 0.9
Quercetin 3-galactoside	134.9 ± 18.5	56.0 ± 6.7	50.3 ± 2.5	100.9 ± 13.1	90.6 ± 8.0
Quercetin 3-glucoside + rutinoside	38.9 ± 3.0	105.7 ± 8.8	40.4 ± 2.2	40.4 ± 3.5	ND
Quercetin 3-xyloside	ND	ND	31.9 ± 1.6	ND	17.9 ± 1.9
Quercetin 3-acetylrhamnoside	22.7 ± 1.1	21.7 ± 3.1	91.5 ± 5.2	46.7 ± 6.2	14.3 ± 1.5
Unknown flavonols	84.9 ± 3.4	49.0 ± 8.0	50.9 ± 2.4	83.0 ± 5.7	23.8 ± 5.6
Total flavonols[c]	327.5a	261.6b	304.3ab	315.9a	172.5c
ORAC[c]	95.9b	51.8c	62.2d	139.4a	85.4c

[a] Data expressed as rutin equivalents.
[b] Breeding selection not available for sale or present in commerce at the time of writing.
[c] Values within a row with similar letters are not significantly different (LSD, $P > 0.05$).
[d] Standard deviation ($n = 3$). ND, not detected.

Table 8. Anthocyanin content (mg kg^{-1} fresh weight[a]) of blackberry genotypes

	Genotype					
Compound	Apache	APF-12[b]	Arapaho	Chickasaw	Kiowa	Navaho
Cyanidin 3-glucoside	1906.2 ± 9.2[d]	1093.9 ± 5.9	1428.3 ± 6.4	852.1 ± 8.7	1583.3 ± 16.5	1459.2 ± 28.6
Cyanidin 3-rutinoside	91.1 ± 0.6	109.8 ± 3.6	134.8 ± 2.9	138.1 ± 2.3	44.5 ± 16.2	13.4 ± 4.7
Cyanidin 3-xyloside	178.1 ± 0.7	57.9 ± 1.1	78.4 ± 0.9	46.0 ± 3.0	76.7 ± 1.3	147.2 ± 1.7
Cyanidin 3-malonylglucoside	46.6 ± 2.4	23.8 ± 6.0	46.7 ± 2.5	35.6 ± 3.4	55.0 ± 0.3	50.4 ± 1.0
Cyanidin 3-dioxalylglucoside	193.4 ± 1.0	35.7 ± 0.4	105.8 ± 1.0	63.0 ± 1.5	117.2 ± 2.4	143.3 ± 3.7
Unknown anthocyanins	ND	4.4 ± 0.1	4.9 ± 2.3	9.1 ± 1.3	9.9 ± 0.3	9.5 ± 0.4
Total anthocyanins[c]	2415.4a	1325.5d	1798.9c	1143.9e	1886.6b	1823.0c

[a] Data expressed as cyanidin 3-monoglucoside equivalents.
[b] Breeding selection not available for sale or present in commerce at the time of writing.
[c] Values with similar letters are not significantly different (LSD, $P > 0.05$).
[d] Standard deviation ($n = 3$). ND, not detected.

A-98, Ozarkblue, US-497 and US-720. In contrast, the percentage distribution of galactosides (35.6%), glucosides (28.7%) and arabinosides (29.3%) was similar in Bluecrop. The higher levels of glucosides and lower levels of galactosides in Bluecrop compared with the other genotypes may be due to higher glucosyl transferase and lower galactosyl transferase activities associated with the variety's pure *V corymbosum* background. In contrast to Bluecrop, all the other genotypes measured have other species in their background and apparently have a greater propensity to synthesise higher levels of galactosides than glucosides.

Total flavonol levels were much lower than levels of total anthocyanins, ranging from a low of 172.5 mg kg^{-1} for US-720 to a high of 327.5 mg kg^{-1} for A-98 (Table 7). The total flavonol content of Bluecrop (261.6 mg kg^{-1}) was lower than the value of 401 mg kg^{-1} reported by Skrede *et al*[19] for Bluecrop. In terms of composition, quercetin 3-galactoside was the predominant flavonol in A-98, US-497 and US-720, quercetin 3-glucoside + rutinoside was the predominant flavonol in Bluecrop and quercetin 3-acetylrhamnoside was the predominant flavonol in Ozarkblue. Low levels of myricetin 3-galactoside/glucoside and myricetin 3-rhamnoside were present in all the genotypes. Quercetin 3-xyloside was present only in Ozarkblue and US-720. Chlorogenic acid was the only hydroxycinnamate detected in the blueberry genotypes. The content of chlorogenic acid ranged from a low of 363 mg kg^{-1} for US-497 to a high of 1082 mg kg^{-1} for A-98 (data not shown). The chlorogenic acid content of Bluecrop (415 mg kg^{-1}) fell within the lower range of values, 274,[19] 977–1009[30] and 1850 mg kg^{-1},[32] previously reported for Bluecrop.

ORAC values of the five genotypes followed a similar trend to total anthocyanins, ranging from a low of 51.8 mmol TE kg^{-1} for Bluecrop to a high of 139.4 mmol TE kg^{-1} for US-497 (Table 7). A-98 (95.9 mmol TE kg^{-1}) and US-720 (85.4 mmol TE kg^{-1}) also had exceptionally high ORAC values. Linear relationships were observed between ORAC values and total anthocyanin monoglycosides ($r_{xy} = 0.94$) and total anthocyanins ($r_{xy} = 0.95$), whereas non-linear relationships were observed

Table 9. Percentage distribution of anthocyanins in blackberry genotypes

Genotype	Cyanidin 3-glucoside	Cyanidin 3-rutinoside	Cyanidin 3-xyloside	Cyanidin 3-malonyl-glucoside	Cyanidin 3-dioxalyl-glucoside	Unk[a]
Apache	78.9	3.8	7.4	1.9	8.0	0.0
APF-12[b]	82.5	8.3	4.4	1.8	2.7	0.3
Arapaho	79.4	7.5	4.4	2.6	5.9	0.3
Chickasaw	74.5	12.1	4.0	3.1	5.5	0.8
Kiowa	83.9	2.4	4.1	2.9	6.2	0.5
Navaho	80.0	0.7	8.1	2.8	7.9	0.5

[a] Unknown peak expressed as cyanidin 3-monoglucoside equivalents.
[b] Breeding selection not available for sale or present in commerce at the time of writing.

Table 10. Flavonol content (mg kg^{-1} fresh weight[a]) and oxygen radical absorbance capacity ($ORAC_{FL}$) (mmol TE kg^{-1} fresh weight) of blackberry genotypes

	Genotype					
Compound/ORAC	Apache	APF-12[b]	Arapaho	Chickasaw	Kiowa	Navaho
Quercetin 3-galactoside	80.4 ± 1.2[d]	12.0 ± 0.6	50.0 ± 1.1	19.4 ± 0.4	50.3 ± 1.0	34.0 ± 0.7
Quercetin 3-glucoside + rutinoside	12.9 ± 0.6	76.1 ± 2.3	20.1 ± 0.5	47.9 ± 1.1	22.5 ± 1.1	26.1 ± 1.1
Quercetin 3-xyloside	ND	11.8 ± 0.4	25.1 ± 0.4	17.6 ± 0.2	11.3 ± 0.1	11.2 ± 0.2
Quercetin 3-xylosylglucuronide	56.9 ± 1.9	10.0 ± 0.1	6.2 ± 0.9	9.7 ± 0.5	34.1 ± 0.2	25.3 ± 0.8
Quercetin 3-glucosylxyloside	ND	9.4 ± 0.2	5.6 ± 0.1	ND	7.2 ± 0.1	5.6 ± 0.5
Unknown flavonols	10.0 ± 0.8	2.0 ± 0.2	9.7 ± 0.4	7.4 ± 0.3	8.0 ± 0.2	8.2 ± 0.8
Total flavonols[c]	160.2a	121.3c	116.7d	102.0f	133.4b	110.4e
ORAC[c]	82.5a	62.5c	71.8b	65.7c	79.5a	82.2a

[a] Data expressed as rutin equivalents.
[b] Breeding selection not available for sale or present in commerce at the time of writing.
[c] Values within a row with similar letters are not significantly different (LSD, $P > 0.05$).
[d] Standard deviation ($n = 3$). ND, not detected.

between ORAC values and total flavonols and chlorogenic acid content. The small-fruited genotypes A-98, US-497 and US-720 had much higher levels of total anthocyanins and ORAC values then the large-fruited genotypes Bluecrop and Ozarkblue. As previously noted,[30] small-fruited berries have a much larger surface area of skin relative to pulp and hence contain more anthocyanins and ORAC on a per kilogram basis, since anthocyanins and flavonols are located predominantly in the skin.

Flavonoid components and antioxidant activities in blackberries

The total anthocyanin content of blackberry genotypes ranged from a low of 1143.9 mg kg^{-1} for Chickasaw to a high of 2415.4 mg kg^{-1} for Apache, reflecting a 2.1-fold difference in total anthocyanin content among genotypes (Table 8). Apache, Navaho and Kiowa had high levels of total anthocyanins (>1800 mg kg^{-1}), while APF-12 (1325.5 mg kg^{-1}) and Chickasaw (1143.9 mg kg^{-1}) had much lower levels. The Cyd 3-glucoside content of the six genotypes followed the same trend as total anthocyanins, with Apache having the highest and Chickasaw the lowest content. In addition to a higher content of Cyd 3-glucoside, Apache also had higher contents of Cyd 3-xyloside and Cyd 3-dioxalylglucoside than the other genotypes. Blackberries were unique compared with grapes and blackberries in that all the anthocyanins detected were monomeric or acylated derivatives of Cyd, indicating that blackberries lack the genetic capacity to synthesise Ptd, Mvd, Pnd and Dpd.

The percentage distribution of anthocyanins in the five genotypes of blackberries is presented in Table 9. Cyd 3-glucoside accounted for 75–84% of total anthocyanins, while Cyd 3-rutinoside (0.7–12.1%), Cyd 3-xyloside (4.0–8.1%), Cyd 3-malonylglucoside (1.8–3.1%), and Cyd 3-dioxalylglucoside (2.7–8.0%) accounted for the remainder of total anthocyanins. The high levels of Cyd glycosides in blackberries may be important in terms of human health benefits owing to their purported antioxidant and anti-inflammatory properties.[33]

Although the blackberry genotypes varied in total flavonol content, the levels were low compared with total anthocyanins, ranging from a low of 102.0 mg kg^{-1} for Chickasaw to a high of 160.2 mg kg^{-1} for Apache (Table 10). Quercetin 3-galactoside was the predominant flavonol in Apache, Arapaho, Kiowa and Navaho, while the sum of quercetin 3-glucoside and quercetin 3-runtinoside was the predominant flavonol in APF-12 and Chickasaw. Arapaho contained the highest amount of quercetin 3-xyloside and Apache contained the highest amount of quercetin 3-xylosylglucuronide. Myricetin was not detected in any of the blackberry genotypes, which

confirms the findings of Bilyk and Sapers.[34] Blackberries appear to be unique in that they only contain derivatives of Cyd and quercetin, indicating that they may lack the genetic capacity to synthesise the enzyme flavonoid-3′5′-hydroxylase which converts dihydrokaempferol to dihydromyricetin, the key step in the biosynthetic pathway for myricetin, Dpd, Ptd and Mvd.[35]

Consistent with results obtained for red wine grapes and blueberries, the ORAC values of the five blackberry genotypes followed the same trend as total anthocyanins, ranging from a low of 62.5 mmol TE kg^{-1} for APF-12 to a high of 82.5 mmol TE kg^{-1} for Apache (Table 10). Navaho and Kiowa also had exceptionally high ORAC values. A linear relationship was observed between ORAC values and total acylated anthocyanins ($r_{xy} = 0.91$), while less linear relationships were obtained between ORAC values and total anthocyanin monoglucosides ($r_{xy} = 0.69$) and total anthocyanins ($r_{xy} = 0.74$). The higher correlation obtained between ORAC and acylated anthocyanins compared with ORAC and total anthocyanin monoglucosides was consistent with results obtained for red wine grapes, indicating that acylated anthocyanins may contribute more to ORAC than monoglucosides. Total flavonols did not correlate well with ORAC.

CONCLUSION

The gradient method developed in conjunction with the Symmetry® C18 column allowed for complete separation and identification of all monomeric anthocyanin glycosides commonly found in blackberries, blueberries and red wine grapes. Also, it showed the possibility of fractionating anthocyanins and flavonols within a single run owing to two distinct elution regions. The specific levels of anthocyanins, flavonols and ORAC varied within genotypes and among fruits. Genotypes of fruit that contained high levels of phenolics, especially anthocyanins, exhibited the highest antioxidant capacity. In blackberries and red wine grapes, acylated anthocyanins correlated more highly with ORAC than anthocyanin monoglucosides, indicating that acylated anthocyanins made a significant contribution to antioxidant activity in the fruit. Genetic differences among the genotypes measured likely accounted for much of the variation in the data. This information provides further encouragement that selection in breeding can be utilised to increase the levels of beneficial compounds in these fruits, and that broadening the species background in the various fruits may be of value as part of the breeding strategy.

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